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The Pharmacological Landscape and Therapeutic Potential of Serine Hydrolases

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Abstract

Serine hydrolases play critical roles in many biological processes, and several are targets of approved drugs for indications such as type 2 diabetes, Alzheimer's disease, and infectious disease. Despite this, most of the 200+ human serine hydrolases remain poorly characterized with respect to their physiological substrates and functions, and the vast majority lack selective, *in vivo*-active inhibitors. Here, we review the current state of pharmacology for mammalian serine hydrolases, including marketed drugs, compounds under clinical investigation, and selective inhibitors emerging from academic probe development efforts. We also highlight recent methodological advances that have accelerated the rate of inhibitor discovery and optimization for serine hydrolases, which we anticipate will aid in their biological characterization and, in some cases, therapeutic validation.

Introduction

Serine hydrolases are one of the largest and most diverse classes of enzymes found in eukaryotes and prokaryotes. These enzymes, which include lipases, (thio)esterases, amidases, peptidases, and proteases, all utilize a base-activated serine nucleophile to cleave amide or (thio)ester bonds in substrates *via* a covalent acyl-enzyme intermediate (Fig. 1). In mammals, serine hydrolases represent ~1% of all proteins and play vital roles in many (patho)physiological processes, including blood clotting[1], digestion[2], nervous system signaling[3], inflammation[4], and cancer[5–7]. Serine hydrolases also perform critical functions in bacteria and viruses, where they contribute to pathogen life cycle[8], virulence[9], and drug resistance[10].

The widespread biological significance of serine hydrolases has motivated many academic and industrial groups to develop inhibitors for enzymes from this class, both for use as chemical probes to study enzyme function and as potentially new therapeutic agents. Four general strategies have been successfully employed: 1) mining natural products (proteins, polysaccharides, and small-molecules), 2) converting endogenous substrates into inhibitors, 3) screening large compound libraries and optimizing lead scaffolds, and 4) tailoring compounds containing mechanism-based electrophiles, including carbamates[11, 12], ureas [13, 14], activated ketones[15], and lactones/lactams[16, 17], that covalently react with the active-site serine nucleophile. Although the last approach overlaps with the other strategies —e.g., electrophiles are intrinsic in some natural product scaffolds[18, 19] and have been extensively employed as "warheads" on enzyme substrates and/or products to convert them into inhibitors[20, 21]—screening of "hydrolase-directed" electrophile libraries broadly against serine hydrolases has emerged as a particularly fruitful independent approach for the identification of inhibitors[11, 14]. Together, these efforts have yielded a diverse array of

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pharmacological tools, including proteins, peptides, polysaccharides, and small-molecules, that inhibit serine hydrolases with good selectivity, and several of these agents have been approved for clinical use to treat diseases such a type 2 diabetes, obesity, blood-clotting disorders, Alzheimer's disease, and bacterial and viral infections [Table 1 and Supplementary information S1 (table)].

Despite these advances, the vast majority of eukaryotic and prokaryotic serine hydrolases still lack selective inhibitors. Further, many serine hydrolases, including some that have been genetically linked to human disease, remain uncharacterized with respect to their physiologic substrates and functions. In this Review, we survey the current pharmacological toolkit and therapeutic potential for human serine hydrolases, giving special attention to modern chemoproteomic methods that have quickened the pace of inhibitor discovery and optimization. We also discuss the challenges that must be overcome to create selective inhibitors for the vast majority, if not all mammalian serine hydrolases, and highlight how they are being met by advances in screening and the development of classes of compounds that show preferential capacity to inactivate serine hydrolases.

The human serine hydrolases

There are ~240 human serine hydrolases, which can be divided into two near-equal-sized subgroups – the serine proteases (~125 members) and the 'metabolic' serine hydrolases (~115 members) (Fig. 2).[6] The vast majority of serine proteases, which primarily cleave peptide bonds in proteins, have chymotrypsin-like or subtilisin-like folds (Fig. 2, black and red enzymes, respectively), with a catalytic serine nucleophile activated by participation in a catalytic triad with conserved histidine and aspartic acid residues.[22] Serine proteases typically exist as inactive precursors (i.e., zymogens), which are activated by limited proteolysis upon specific biological stimuli and subsequently inactivated by endogenous protein inhibitors.[22, 23] These enzymes include the well studied digestive protease trypsin and the critical blood clotting mediators thrombin and activated factor Xa (FXa).

The 'metabolic' serine hydrolases (Fig. 2) are comprised of a wide range of lipases, peptidases, esterases, thioesterases, and amidases that hydrolyze small-molecules, peptides, or post-translational (thio)ester protein modifications.[6] Consistent with their diverse substrate repertoire, the metabolic serine hydrolases are comprised of a much more structurally diverse group of enzymes than the serine proteases (Fig. 2, note branch length). The majority (>60%) of metabolic serine hydrolases have an α/β-hydrolase fold and Ser-His-Asp catalytic triad, but this sub-family also includes several structurally and mechanistically distinct enzyme clades such as the patatin domain-containing lipases[24] and the amidase signature enzymes[25, 26], which use Ser-Asp dyads and Ser-Ser-Lys triads for catalysis, respectively. Although several members of the metabolic serine hydrolase family have been extensively characterized, including acetylcholinesterase (ACHE), fatty acid amide hydrolase (FAAH), and dipeptidyl peptidase-4 (DPP-4), the majority are still unannotated with respect to their physiological substrates and functions.[6]

Clinically approved inhibitors of human serine hydrolases

Small-molecule inhibitors have been clinically approved for six distinct human serine hydrolase targets, four of which are described below (Table 1). As several of these compounds are not perfectly selective for a single enzyme, and at least one, orlistat, is thought to derive therapeutic benefit from inhibiting several related enzymes[27], the actual number of human serine hydrolases targeted by commercial drugs is likely higher than six. Interestingly, despite the pharmaceutical industry's perceived aversion to developing therapeutics that form covalent bonds with protein targets[28], five of these drugs, rivastigmine[29], saxagliptin[30], vildagliptin[31], orlistat[32], and sivelestat[33, 34], contain

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electrophilic chemical groups that interact covalently with their target's active-site serine nucleophile. Additional examples of electrophilic drugs that target serine hydrolases include the β-lactam antibiotics[35], which inhibit bacterial transpeptidase and β-lactamase enzymes, and the recently approved hepatitis C virus (HCV) drugs boceprevir and telaprevir[36], which are α-keto amides that inhibit the HCV NS3 protease [Supplementary information S1 (table)]. In addition to these small-molecule inhibitors, several large biomolecules and their derivatives (proteins, peptides, polysaccharides) that target serine hydrolases, such as thrombin, are either in clinical development or have been approved for clinical use[37]. However, due to space limitations, we will focus on small-molecule inhibitors of human serine hydrolases in this Review.

Inhibitors of serine proteases involved in coagulation

Venous and arterial thromboembolic diseases, which are characterized by occlusion of blood vessels by thrombi (i.e., aggregations of platelets, fibrin, and cells), are a major cause of morbidity and mortality worldwide[37]. Several serine proteases play central roles in the blood coagulation pathway, where sequential activation of protease zymogens results in the rapid formation of insoluble fibrin blood clots[1, 38], and have long been the main targets of anticoagulant drug development efforts. For the past half-century, heparins and vitamin K antagonists (e.g. warfarin), both of which indirectly inactivate several proteases in the cascade, have been the two major anticoagulant drug classes. However, these agents have important clinical drawbacks; heparins require parenteral administration due to their large size, and warfarin, although orally available, has a narrow therapeutic window, many food-drug interactions, and requires frequent monitoring[37]. More recent research efforts have focused on the development of selective and orally available small-molecules that directly block one of two key coagulation proteases, thrombin (also known as factor IIa) and activated factor Xa (FXa).

Thrombin, the final protease in the clotting cascade, cleaves fibrinogen into fibrin, potently activates platelets, and indirectly activates itself through a feedback loop[39]. Injectable direct thrombin inhibitors (DTIs) have been known for many years; the leech salivary peptide hirudin[40], the hirudin-derivative bivalirudin[41], and the small-molecule argatroban[42] (Table 1) are all clinically approved DTI anticoagulants[43]. The first orally available small-molecule DTI, ximelagatran (Exanta; AstraZeneca) was developed starting from a peptide scaffold that mimicked the thrombin substrate fibrinogen[39]. Ximelagatran, however, exhibited serious liver toxicity, and consequently was not approved in the United States and was withdrawn in Europe in 2006[44]. The next attempt to develop an orally available DTI originated from an X-ray crystal structure of the peptide-like inhibitor NAPAP in complex with bovine thrombin[45]. Replacement of the central NAPAP glycine residue with a more rigid isostere and subsequent optimization resulted in the reversible inhibitor dabigatran, which exhibited excellent anticoagulant activity in human blood with good selectivity for thrombin over related serine proteases[46]. However, dabigatran was not orally bioavailable, likely due to a highly basic amidine residue that was included to mimic the fibrinogen substrate. In order to achieve oral bioavailability, dabigatran was masked as a double prodrug (Table 1) (Dabigatran etexilate; Pradaxa; Boehringer Ingelheim), which is hydrolyzed to release dabigatran *in vivo*[47]. Importantly, dabigatran etexilate did not show any evidence of liver toxicity[48], and has recently gained regulatory approval worldwide.

FXa, the other major protease target for anticoagulant development, cleaves prothrombin into active thrombin[49]. Potent parenteral FXa inhibitors have been known for decades, including the polypeptides antistasin[50, 51] and the tick anticoagulant peptide (TAP)[52]. These agents, together with more recently introduced pentasaccharide fondaparinux (Arixtra; GlaxoSmithKline)[53], an analog of the heparin core that selectively inhibits FXa but not thrombin, were critical in elucidating the role of FXa in thrombosis and validating selective

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FXa inhibition as a therapeutic strategy[54]. Initial small-molecule FXa inhibitors all contained amidine residues that served as prothrombin mimetics[55, 56]. As was observed with dabigatran, this highly basic group, although critical for potency, contributed to poor oral bioavailability[57, 58]. Bayer opted instead to screen a large (~200,000) library of compounds to identify a novel inhibitor scaffold[59]. From a lead with modest micromolar potency and structure-activity knowledge emanating from previous efforts, this team developed the highly potent, reversible FXa inhibitor rivaroxaban (Xarelto; Bayer HealthCare)[54, 59]. Rivaroxaban became the first selective small-molecule inhibitor of FXa approved for clinical use in 2008[54]. Several additional small-molecule inhibitors of both thrombin and FXa are currently in clinical development and have been recently reviewed[60].

Acetylcholinesterase inhibitors to treat Alzheimer's associated dementia

Acetylcholinesterase (ACHE) is a metabolic serine hydrolase that cleaves and inactivates the neurotransmitter acetylcholine[61]. More than 30 years ago, a decrease in cholinergic signaling was first observed in patients with Alzheimer's disease[62–64], leading to the hypothesis that a loss in cholinergic neurotransmission contributed to the decline in cognitive function in these patients[65]. Consequently, it was proposed that increasing acetylcholine levels by inhibiting ACHE could alleviate symptoms of this disease. This premise has been validated clinically, and three ACHE inhibitors are currently used for the treatment of Alzheimer's associated dementia (Table 1). A fourth inhibitor, tacrine (Cognex; Shionogi), is approved but not recommended for use due to poor bioavailability and toxicity[66]. Essential to the successful use of these drugs is a graduated dosing regimen that avoids overt cholinergic toxicity[67], such as that observed with large doses of organophosphorus nerve agents and insecticides that potently, but nonselectively inhibit ACHE[68].

The three ACHE inhibitors in clinical use have notably different origins. Only one of these compounds, donepezil (Aricept; Eisai), is entirely synthetic, a result of derivatization of a scaffold identified from "blind" compound screening[69]. Donepezil reversibly inhibits ACHE, and has the highest selectivity (>1,000 fold) of the approved compounds for ACHE over the related serine hydrolase butyrylcholinesterase (BCHE)[70, 71]. Galantamine (Razadyne; Ortho-McNeil Janssen) is a natural product alkaloid first isolated in 1952 from the bulbs of the Caucasian snowdrop *Galanthus woronowi*[72]. Like donepezil, galantamine is a reversible inhibitor, but has a more modest 50-fold selectivity for ACHE over BCHE[73, 74]. The third approved compound, rivastigmine (Exelon; Novartis), is an optimized version of physostigmine, a natural product alkaloid with cholinergic activity[75], with improved selectivity for the brain isoform of ACHE over peripheral ACHE and BCHE[76]. Rivastigmine, like physostigmine, contains an aryl carbamate group that acts as a slowly turned over ACHE substrate, effectively leading to the irreversible inactivation of the enzyme[29]. Following on the success of rivastigmine, carbamates have emerged as a versatile chemotype for serine hydrolase inhibitors, as embedding this tempered reactive group into various structural scaffolds has generated selective inhibitors for a diverse number of serine hydrolases[11, 77, 78], as described in more detail below.

Dipeptidyl peptidase 4 (DPP-4) inhibitors for type 2 diabetes

Dipeptidyl peptidase 4 (DPP-4) is a serine peptidase that cleaves N-terminal dipeptides from a variety of polypeptides that contain a proline or an alanine residue at the penultimate position[79]. Prominent among DPP-4 substrates are the incretins glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), which are released from the gut after food intake to promote insulin secretion and improve pancreatic β cell function[80–84]. Inhibition of DPP-4 prolongs the beneficial actions of GLP-1 and GIP, designating this enzyme as a therapeutic target for the treatment type 2 diabetes[85].

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Since 2007, five DPP-4 inhibitors have been approved for clinical use (Table 1), although vildagliptin[31] (Zomelis; Novartis) and alogliptin[86] (Nesina; Takeda) have not been approved in the United States. The earliest reported DPP-4 inhibitors were proline (or alanine)-based dipeptides (i.e., analogs of DPP-4 cleavage products) bearing chemical warheads, including boronic acids[21], diphenyl phosphonates[87], and nitriles[20]. Appropriately positioned nitrile groups, in particular, which form covalent reversible bonds with the serine nucleophile of DPP-4 to give high affinity binding[30, 88, 89], resulted in selective and orally bioavailable compounds. These lead compounds were ultimately optimized to give vildagliptin[31, 90] and saxagliptin [91, 92] (Onglyza; Bristol Myers Squibb). Sitagliptin[93] (Januvia; Merck) and linagliptin[94] (Tradjenta; Boehringer Ingelheim), in contrast, were both optimized from novel structures—a β-amino acid scaffold[95] and xanthene-based scaffold[94], respectively—identified from screening compound libraries. Finally, alogliptin emerged from medicinal chemistry efforts around a quinazolinone scaffold predicted to inhibit the active-site of DPP-4 by structure-based design[86]. Sitagliptin, linagliptin, and alogliptin inhibit DPP-4 through non-covalent, reversible mechanisms.

Human serine hydrolases with emerging therapeutic potential

Many additional members of the serine hydrolase class have been implicated in disease, and inhibitors for several of these targets are in clinical development (Table 2). For example, fatty acid amide hydrolase (FAAH) inactivates a large class of amidated lipid transmitters, including the endogenous cannabinoid anandamide[96]. Genetic deletion or chemical inactivation of FAAH in rodents increases the levels of anandamide and related fatty acid amides to produce analgesia, anti-inflammation, anxiolysis, and anti-depression without the psychotropic side effects typically observed with direct cannabinoid receptor (CB1) agonists[77, 97, 98]. Recently, a high-throughput screen of the Pfizer chemical library uncovered a novel urea-containing FAAH inhibitor, which irreversibly inactivates the enzyme by covalently modifying FAAH's active-site serine[99]. The subsequent optimization of this scaffold resulted in the discovery of PF-04457845 (Table 2)[13], a urea with exceptional selectivity for FAAH over other serine hydrolases and excellent pharmacokinetic properties in rats and dogs. The oral administration of PF-04457845 at 0.1 mg/kg exhibited similar antihyperalgesic effects as naproxen at 10 mg/kg in a rat model of inflammatory pain, and has since entered clinical trials.

A second example of an emerging drug target in the serine hydrolase class is PLA2G7 (or Lp-PLA2), a calcium-independent phospholipase A2 principally secreted by leukocytes and associated with circulating low density lipoprotein (LDL)[100]. Elevated levels of PLA2G7 were discovered to strongly correlate with an increased risk of coronary heart disease, suggesting a potential role for this enzyme in atherogenesis[101]. PLA2G7 can hydrolyze polar phospholipids in oxidized LDL to generate two key pro-inflammatory mediators, lysophosphatidylcholine (LPC) and oxidized nonesterified fatty acids (NEFAs)[102, 103]. LPC and oxidized NEFAs have been implicated in the development of atherosclerosis through several mechanisms, including homing of inflammatory cells and induction of apoptosis[100]. To investigate the biology and therapeutic potential of PLA2G7, GlaxoSmithKline optimized a selective, picomolar PLA2G7 inhibitor, darapladib (Table 2)[104], from an initial micromolar HTS screening hit[105]. Darapladib blocked LPC and NEFA production in oxidized LDL[103] and significantly decreased coronary atherosclerotic plaque development in a diabetic and hyperchloesterolemic swine model through an anti-inflammatory mechanism independent of cholesterol[106]. Darapladib is currently being evaluated in Phase III clinical trials.

More preliminary studies using gene knockouts, lead chemical inhibitors, and protein and gene expression profiling have implicated other serine hydrolases as being of potential therapeutic importance. For example, mice lacking prolylcarboxypeptidase (PRCP), which

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cleaves C-terminal amino acids after proline in bioactive peptides, including angiotensin II and III[107], have reduced body weight, food intake, and fat mass, designating PRCP as a potential target for obesity[108]. Merck has reported initial attempts to develop PRCP inhibitors[109, 110], the first of which, 'compound 8o'(Table 2), reversibly blocked PRCP activity with nanomolar potency and high selectivity over a panel of related serine peptidases[109]. Encouragingly, 'compound 8o' significantly reduced food intake, body weight, and fat mass of wild-type mice compared with PRCP$^{-/-}$ mice. However, a recently reported second generation PRCP inhibitor reduced body weight and food intake similarly in wild-type and PRCP$^{-/-}$ mice[110], indicating the effects, at least in this case, were independent of PRCP. The authors speculate that 'compound 8o' may achieve higher levels of peripheral and/or central PRCP engagement than the second generation compound, and are currently pursuing more structurally diverse inhibitors with improved bioavailability to further evaluate this premise.

In addition, murine knockouts of three serine triglyceride (TG) hydrolases, triacylglycerol hydrolase (TGH), adipose triglyceride lipase (ATGL), and endothelial lipase (LIPG or EL), have implicated these enzymes as possible therapeutic targets for hypertriglyceridemia, cancer-associated cachexia, and cardiovascular disease, respectively. TGH, also referred to as carboxylesterase 3 (CES3) in mice and carboxylesterase 1 (CES1) in humans, can cleave TG stores in hepatocytes, which, after lipolysis, can serve as substrates for the assembly of apolipoprotein B (apoB)-containing very low-density lipoprotein (VLDL) particles[111–113]. Excitingly, TGH$^{-/-}$ mice have significantly decreased plasma triacylglycerol and apoB levels accompanied by improved insulin sensitivity and glucose tolerance[114]. GlaxoSmithKline has introduced the TGH inhibitor GR148672X (Table 2)[115], but the selectivity, bioavailability, and molecular interactions of this compound with TGH have not been disclosed. ATGL, which can also mediate the lipolysis of stored TGs[116], was recently evaluated for its role in an animal model of cancer-associated cachexia (CAC)[117], a wasting syndrome characterized by the uncontrolled loss of muscle and adipose tissue. In this model, ATGL$^{-/-}$ mice resisted the loss of white adipose tissue and muscle mass observed in wild-type mice, suggesting ATGL inhibition could slow or stop CAC progression. However, to our knowledge ATGL inhibitors have not yet been reported. LIPG is an extracellular TG lipase that also possesses significant phospholipase activity[118]. LIPG$^{-/-}$ mice have increased high-density lipoprotein (HDL) levels compared to wild-type mice[119, 120], whereas mice with transgenic overexpression of LIPG have significantly reduced HDL levels[120]. As HDL levels are inversely correlated with risk of cardiovascular disease, these genetic models strongly suggest that LIPG is a potential therapeutic target for this indication. GlaxoSmithKline has reported initial sulfonylfuran urea-based LIPG inhibitors (Table 2), but these compounds also inhibit the related enzyme lipoprotein lipase (LPL) and have not yet been evaluated *in vivo*[121].

Another potential serine hydrolase drug target is prolyl endopeptidase (PREP), which is also referred to as prolyl oligopeptidase (POP). PREP is a post-proline cleaving enzyme that is highly expressed in the brain, kidney, and muscle, and testes[122], and can degrade a variety of neuroactive peptides, including arginine-vasopressin (AVP), substance P, and thyrotropin-releasing hormone, among others[123]. As several of these substrates are involved in learning and memory, the inhibition of PREP has been suggested as a strategy for the treatment of cognitive defects associated with Alzheimer's disease, Parkinson's disease, and aging[124]. The majority of PREP inhibitor discovery efforts have centered around the modification of Z-prolyl-prolinal (ZPP), a peptide-based transition-state analog that competitively inhibits PREP[125]. Two compounds that emerged from this approach, JTP-4819[126] and the S 17092[127] (Table 2), inhibit PREP selectively over related peptidases and elevate the levels of several PREP peptide substrates in the brains of compound treated-animals[126, 128–130]. Encouragingly, the inhibition of PREP has been shown to produce gains

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in cognitive function in aging rats[131] and in a chemically-induced model of early Parkinsonism in monkeys[132]. In humans, S 17092 showed preliminary evidence of eliciting improvements in a delayed memory task and in mood stabilization[133, 134]. Current work focused on the continued development of inhibitors with improved bioavailability and potency, combined with detailed mechanistic studies to molecularly understand the cognition enhancing effects of these compounds, should clarify the therapeutic potential of this target[135].

Multiple serine hydrolases, such as fatty acid synthase (FASN)[5], protein methylesterase-1 (PME-1)[136], and the urokinase-type (uPA) and tissue-type (tPA) plasminogen activators[137], have also been implicated in cancer[138]. Especially intriguing among potential cancer targets is fibroblast activation protein (FAP), the serine peptidase most homologous to DPP-4 (Fig. 2), which is highly expressed by stromal fibroblasts in most epithelial cancers[139–141]. Transfection of FAP in cancer cells promotes tumor growth in animals[142]. The nonselective dipeptide boronic acid FAP inhibitor PT-100 (Table 2) (Talabostat; Point Therapeutics) slowed tumor growth in mice, but PT-100's lack of specificity precludes assignment of the precise role of FAP inhibition in this model[143, 144]. A recent study demonstrated that removing the FAP$^+$ subpopulation of tumor stromal cells arrests the growth of solid tumors by inducing an immune response[145]. The pursuit of selective FAP inhibitors should further elucidate the role that this enzyme plays in tumorigenesis[146, 147].

Activity-based protein profiling for target discovery

As noted above, many serine hydrolases are regulated by post-translational mechanisms, which means that changes in their activity may not correlate well with their expression levels as measured by conventional proteomic[148–151] or genomic[152, 153] methods. This problem has been addressed by the development of a chemoproteomic technology termed activity-based protein profiling (ABPP)[154, 155], which utilizes small-molecule probes to record changes in enzyme activity directly in native biological systems. An activity-based chemical probe typically contains at least two key features: 1) a reactive group that binds and covalently modifies the active sites of a large number of enzymes that share conserved mechanistic and/or structural features, and 2) a reporter tag (e.g., a fluorophore or biotin) to enable detection, enrichment, and identification of probe-labeled enzymes (Fig. 3a). Activity-based probes have been developed for numerous enzyme classes, including serine hydrolases[156], cysteine-dependent enzymes[157–159], kinases[160], and histone deacetylases (HDACs)[161, 162]. Importantly, ABPP can be applied to any biological sample (cell line, tissue, or fluid) and coupled with either gel- or mass spectrometry (MS)-based readouts to characterize numerous enzyme activities in parallel[155]. The most commonly employed activity-based probes for serine hydrolases contain a fluorophosphonate (FP) reactive group that covalently reacts with the conserved serine nucleophile of these enzymes (Fig. 3b). A recent global analysis of tissue and cell line proteomes demonstrated that > 80% of mammalian metabolic serine hydrolases react with FP probes[11]. Although it is considerably more challenging to perform an equivalent survey of the serine proteases, which typically exist endogenously in their inactive zymogen or inhibitor-bound forms, many of these proteases have also been demonstrated react with FP-probes[163–166].

ABPP has been applied to discover serine hydrolases that are involved a wide range of biological processes, including cancer[138], nervous system signaling[167], immune cell function[168], obesity[169, 170], and infectious disease[171]. For example, ABPP studies first identified AADACL1 as highly elevated in aggressive cancer cells[172], where it functions as a 2-acetyl monoalkylglycerol ether (MAGE) hydrolase that controls the levels of the MAGE class of neutral ether lipids (NELs)[173]. Stable knockdown or chemical inhibition (with the carbamate inhibitors AS115 or JW480, Table 3) of AADACL1 in cancer cells reduces the levels of MAGEs and downstream bioactive lysophospholipids, ultimately impairing cancer

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cell migration and invasion and in vivo tumor growth[173, 174]. In addition, monoacylglycerol lipase (MGLL), which can cleave a variety of monoglycerides into free fatty acids and glycerol, was similarly discovered to be highly elevated in aggressive cancer cells and primary tumors[7], where it controls a free fatty acid (FFA) network that includes many oncogenic signaling lipids. Overexpression of MGLL in nonaggressive cancer cells promotes their pathogenicity, whereas knockdown or inhibition of MGLL with the selective inhibitor JZL184[78] (Table 3) impairs of tumor growth[7, 175]. In addition to its potential role in cancer, MGLL is responsible for the hydrolysis and inactivation of the endocannabinoid 2-arachidonoylglycerol (2AG), an endogenous ligand for the cannabinoid receptors CB1 and CB2. Acute MGLL inhibition with JZL184 results in CB1-dependent hypomotility and antinociception[176, 177], suggesting MGLL inhibition could also be a therapeutic strategy for the treatment of pain. It should be noted that ABPP not only played an important role in discovery of AADACL1 and MGLL as therapeutic targets, but also that a competitive version of ABPP, described below, was instrumental in the development of AADACL1 and MGLL inhibitors. Moreover, ABPP analysis has recently shown that the retinoblastoma-binding protein 9 (RBBP9), which was initially discovered as a protein that could override TGF-β-mediated antiproliferative signaling[178], exhibits elevated activity in tumors and promotes anchorage-independent growth and pancreatic carcinogenesis[179]. Preliminary lead inhibitors for RBBP9 have been recently discovered using the high-throughput competitive ABPP strategies described below[180, 181]. Further research into other enzymes identified in ABPP studies, many of which remain poorly characterized, should yield insights into their basic biological functions and reveal whether they possess clinical relevance.

Promising new strategies for inhibitor discovery

Main challenges

Selective chemical inhibitors are notably lacking for the vast majority of human serine hydrolases, hindering investigations of their physiological roles and relationships to human disease. The dearth of inhibitors is likely due to at least three reasons: 1) many enzymes, in particular for those that are poorly characterized, lack suitable activity assays to enable compound screening, 2) achieving inhibitor selectivity for one enzyme is difficult amongst such a large, related protein family, and 3) compound libraries often do not contain tractable starting points for inhibitor optimization. Below, we discuss recently introduced approaches to address these challenges.

Gel-based competitive ABPP

When applied in a competitive format that assays the ability of compounds to block probe labeling of enzymes, ABPP enables inhibitor discovery for enzymes independent of their degree of functional annotation[15, 182, 183]. Competitive ABPP traditionally involves incubation of a native proteome with a small-molecule, followed by labeling with a fluorescent activity-based probe, separation of proteins by SDS-PAGE, and quantification of the fluorescence intensity of probe-labeled enzymes relative to a control proteome (Fig. 3c; "gel-based competitive ABPP").[12, 15] A complementary MS-based platform termed competitive ABPP-MudPIT has been introduced to further enhance the total protein coverage of these experiments[12, 78] (Fig. 3c). ABPP-MudPIT experiments require larger quantities of sample and more time than gel-based ABPP and are therefore typically reserved for more in-depth analysis of interesting lead inhibitors. In either gel or MS formats, a key advantage of competitive ABPP is that it permits simultaneous optimization of both the potency and selectivity of inhibitors against numerous related enzymes without requiring protein purification.

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Gel-based competitive ABPP screening has enabled the discovery of selective inhibitors for many serine hydrolases (Table 3), including MGLL[78], ABHD6[11, 12], and FAAH[15, 184] (Table 2, OL-135). Key to the success of these efforts was the iterative optimization of compounds containing mechanism-based electrophiles, including carbamates, ureas, and activated ketones. Recently, to expand the number of enzyme-inhibitor interactions evaluated by competitive ABPP, a library of ~150 carbamates was screened against a representative 72-member panel of metabolic serine hydrolases[11]. Lead compounds were identified for numerous serine hydrolases, including currently pursued drug targets (e.g., PLA2G7, FAAH) and uncharacterized enzymes (e.g., ABHD11). Two compounds were subsequently optimized to create the first potent, selective, and *in vivo*-active inhibitors for the poorly characterized enzymes ABHD11[11] and AADACL1[11, 174]. Interestingly, this global analysis also identified several unanticipated pharmacological crosspoints within the serine hydrolase class, where two enzymes distantly related by sequence were found to share inhibitor sensitivity profiles. Such findings underscore the value of chemoproteomic methods like competitive ABPP that can assess compound selectivity broadly across an entire enzyme class. Some clades of enzymes (e.g., the dipeptidyl peptidases), however, were not inhibited by any of the tested carbamates, indicating that additional chemotypes may be necessary to successfully target most/all subsets of serine hydrolases. Consistent with this premise, competitive ABPP analysis of a library of triazole ureas recently identified selective inhibitors for enzymes that were insensitive to carbamates, including platelet-activating factor acetylhydrolase-2 (PAFAH2) and acyl-peptide hydrolase (APEH) (Table 3)[14].

Competitive ABPP was also employed to assess the selectivity of the urea FAAH inhibitor PF-04457845 before this compound entered clinical trials[13]. PF-04457845, as well as other urea inhibitors of FAAH[185], irreversibly inactivate this enzyme by carbamylating its serine nucleophile. Even though there are many irreversibly acting drugs on the market today, the pharmaceutical industry tends to prefer non-covalent, reversible inhibitors in large part due to concerns that irreversible inhibitors will lack specificity for a single protein target[28]. This concern, however, can be directly addressed by competitive ABPP and related chemoproteomic methods[186], and when these approaches were applied to urea inhibitors of FAAH, such as PF-04457845, the compounds were found to be extremely selective for FAAH relative to serine hydrolases (and the rest of the proteome)[13, 185]. Once such a high degree of selectivity has been established for irreversible inhibitors, their distinctive advantages over reversibly acting compounds can be highlighted, namely that they can often inactivate their protein targets at very low doses (and with suboptimal pharmacokinetic properties) *in vivo*[28, 186, 187]. Moreover, the enzyme itself serves as a biomarker for compound activity in that *in vivo* inhibition can be experimentally verified by ABPP of cell, tissue, or fluid proteomes from compound-treated animals[78].

High-throughput screening by competitive ABPP

Competitive ABPP has traditionally been limited by throughput, as only a few hundred compounds can be reasonably screened by gel-based methods. To overcome this barrier, a high-throughput version of competitive ABPP has recently been introduced where the interaction between an activity-based probe and an enzyme is monitored by fluorescence polarization (fluopol-ABPP)[180] (Fig. 4). Fluopol-ABPP has been successfully applied to numerous enzymes from multiple mechanistic classes[181, 188, 189], including several serine hydrolases. In one example, protein methylesterase-1 (PME-1), a serine hydrolase that removes an unusual post-translational carboxymethylation from the C-terminus of PP2A[190] and has been implicated in Alzheimer's disease[191] and cancer[136], was screened against the NIH-300,000+ small-molecule library[189, 192]. This effort uncovered an aza-β-lactam inhibitor (ABL127; Table 3) that selectively and covalently inhibits PME-1 with nanomolar

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potency. ABL127, without any medicinal chemistry optimization, showed excellent activity in living cells and mice, where it selectively inhibited PME-1 relative other serine hydrolases and decreased the levels of demethylated PP2A. Interestingly, ABL127 originated from an academic contribution to the NIH library from a synthetic chemistry laboratory exploring the substrate scope of a chiral catalyst[193]. That ABL127 was contributed to the NIH library without any specific protein target in mind underscores the potential of academic chemistry, when paired with high-throughput screening, to serve as a driving force for the discovery of new bioactive chemotypes and chemical probes.

Substrate activity screening

Even though competitive ABPP platforms address several critical challenges in serine hydrolase inhibitor discovery, many enzymes still do not have lead inhibitors, perhaps due to the absence of suitable lead compounds in chemical libraries. This deficiency should be met, at least in part, by the introduction and exploration of new hydrolase-directed chemotypes, as described above. In a complementary strategy, Ellman and coworkers introduced a technique called 'substrate activity screening' (SAS). This method involves the screening of a diverse library of *N*-acyl aminocoumarins, which fluoresce when cleaved, to identify initial, weak-binding enzyme substrates[194]. After a substrate is optimized for improved binding, the cleaved bond can then be replaced with a mechanism-based pharmacophore to covert it directly into an inhibitor. Although SAS has been successfully applied to select cysteine[194, 195] and serine (chymotrypsin)[196] proteases, it requires some intrinsic activity on at least one member *N*-acyl aminocoumarin library, which not all serine hydrolases will likely possess. One potential future direction could involve the creation of an even more diverse *N*-acyl aminocoumarin compound library for initial substrate screening. Alternatively, poor enzyme substrates, including those without any intrinsic fluorescence, can also be identified by other methods, for example by appearing as weak inhibitors in competitive ABPP experiments[181]. These substrates could potentially be converted into effective inhibitors *via* the introduction of appropriate chemical warheads in a similar manner.

Serine traps as 'tethers' for starting point discovery

Electrophilic traps, regardless of their inherent capacity for enzyme selectivity and/or bioavailability, can also be employed as 'tethers' to enable the initial identification and optimization of lead compound scaffolds that would otherwise be too weak-binding to detect. For example, Merck researchers screened a library of α-keto heterocyles, a chemotype well known to inhibit serine hydrolases[15, 197], to achieve a starting point for the development of PRCP inhibitors[109]. After some compound optimization, the α-keto heterocyle group was replaced with an isostere to improve the selectivity for PRCP over related enzymes, avoid potential bioavailability liabilities due to the ketone moiety, and facilitate derivative synthesis. Similarly, an electrophilic ketone was used in the early stages in the development of ximeligatran[39]. We should note that a conceptually analogous strategy, called "tethering", which involves the engineering of a cysteine residue into a protein (or utilizing a native cysteine, if one exists) that can capture weak-binding, disulfide-containing compounds, has shown promise for the identification of starting points for ligand development for several protein classes[198, 199], and could also prove useful for the discovery of serine hydrolase inhibitors.

Engineering of biologics

While the inhibition of intracellular serine hydrolases is still the exclusive domain of small-molecules, some extracellular serine hydrolases may also be targeted by large macromolecules (e.g., hirudin and TAP). Encouragingly, the engineering of such macromolecular protease inhibitors has already been successful in improving existing and

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uncovering novel pharmacological tools[41, 200]. For example, Craik and coworkers used phage display to optimize the *E. coli* protein ecotin, which naturally inhibits several trypsin-fold proteases, for selective inhibition of plasma kallikrein[201]. Similarly, Kunitz domain-containing proteins have been modified for selective blockade of plasma kallikrein[200] and tissue factor-factor VIIa (TF-FVIIa)[202]. In addition to natural protein scaffolds with intrinsic protease inhibitor activity, antibodies have been elicited to selectively block the activity of certain serine proteases[203–205]. One advantage such protein scaffolds offer is the ability to inhibit regions of the protein outside of the enzyme active-site, which are traditionally challenging to bind with small-molecules[205].

Conclusions

Serine hydrolases have already yielded numerous targets of marketed drugs to treat a wide array of human diseases. Given this precedent, it is tantalizing to extrapolate that many additional drug targets may be found among the numerous enzymes from this class that remain poorly characterized. Achieving this goal, however, will require much more extensive efforts to elucidate the biochemical and physiological roles, as well as disease-relevance for serine hydrolases. Here, we believe that the development of selective and *in vivo*-active inhibitors is critical. Indeed, many serine hydrolases play complex roles in mammalian physiology that cannot easily be modeled in cell culture experiments. Consider, for instance, the regulation of the GLP-1 incretin by DPP-4 or the termination of cholingeric and endocannabinoid signaling in the nervous system by ACHE and FAAH, respectively. These pathways, and likely many others regulated by serine hydrolases, require the integrated physiology of an intact animal for their characterization.

So far, *in vivo*-active inhibitors are available for only a handful of serine hydrolases. While it might be tempting to prioritize, based on current biological knowledge, enzymes from the class for future inhibitor development efforts, we would urge against this inclination. As has been nicely delineated in a recent Perspective by Edwards and colleagues, there appears to be a strong correlation between the volume of research activity (and biological understanding) on a particular protein and the availability of high-quality chemical tools to probe this protein's function[206]. This type of meta-analysis suggests that the creation of inhibitors often *precedes and drives* our biological understanding of enzymes, rather than the other way around, and argues for the development of inhibitors for all enzymes, regardless of their current perceived biological and/or therapeutic importance.

Encouragingly, inhibitors emerging from competitive ABPP span the full range of serine hydrolases to include enzymes that are biologically characterized and those that are devoid of functional annotation. Continued efforts following the inhibitor discovery strategies described in this Review, in particular, high-throughput screening of compound libraries and diversification of mechanism-based electrophiles, show particular promise, in our mind, to deliver new chemical probes for serine hydrolases. We also believe that further expansion of the NIH small-molecule library with structurally diverse compounds, like the aza-β-lactams, should provide useful new starting points for drug development, as well as an exciting opportunity for academic synthetic chemists. While achieving the ultimate goal of developing a selective and *in vivo*-active inhibitor for every mammalian serine hydrolase (as well as critical serine hydrolases in important human pathogens) may seem far away, we believe that it can be accomplished. The resulting pharmacopeia would not only power biological discovery, but also serve as a starting point for next-generation therapeutics for the betterment of human health.

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Supplementary Material

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Acknowledgments

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Figure 1. Schematic representation of the serine hydrolase catalytic cycle

A base-activated serine nucleophile attacks the carbonyl carbon of the scissile bond, forming a covalent intermediate and releasing the first reaction product. A water molecule then hydrolyzes the covalent intermediate to release the second reaction product and regenerate the active enzyme. X= N, O, or S.

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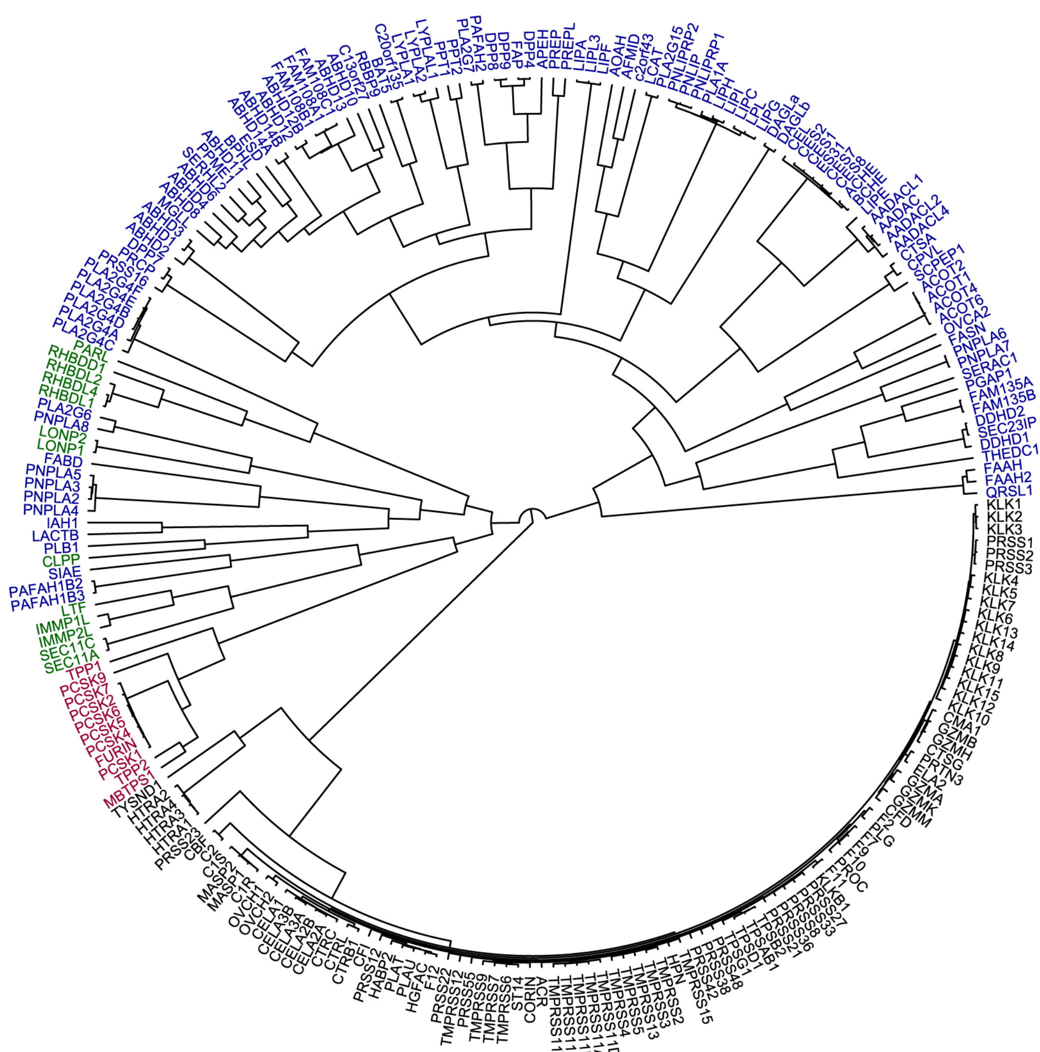

Figure 2. The human serine hydrolases

A dendrogram showing the ~240 predicted human serine hydrolases with branch length depicting sequence relatedness. The metabolic serine hydrolases are colored blue. The remaining enzymes are serine proteases, with chymotrypsin-like enzymes colored black, subtilisin-like enzymes colored red, and other, smaller serine protease clans colored green.

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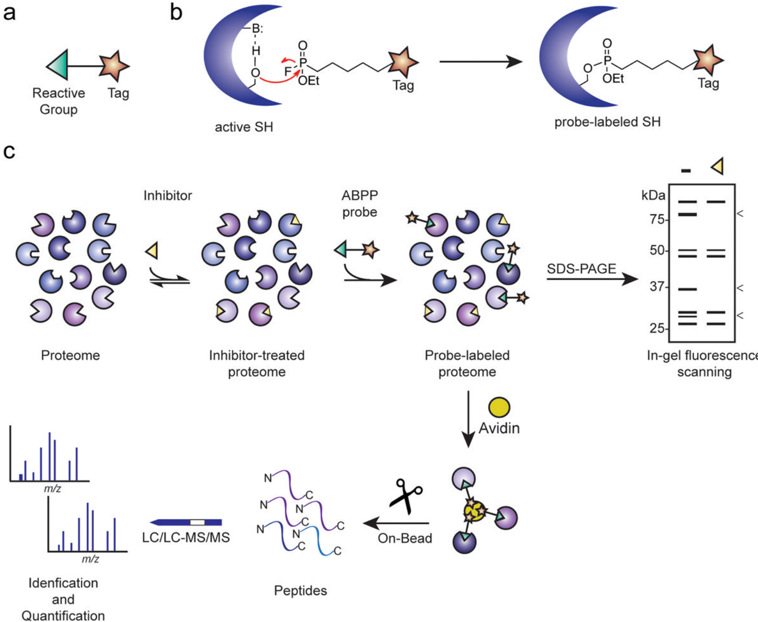

Figure 3. Overview of the current pharmacological toolkit for serine hydrolases
The electrophilic moieties of each compound, if applicable, are colored red.

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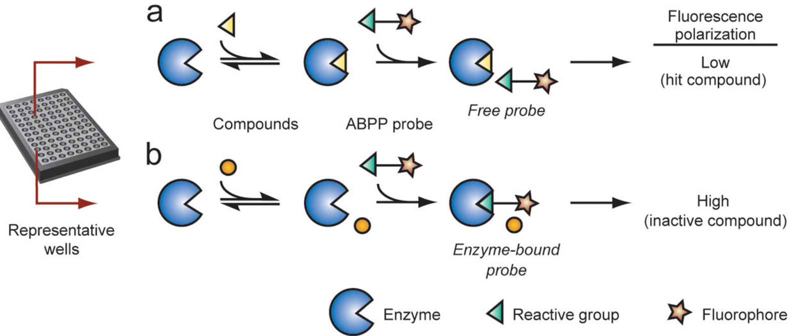

Figure 4. Activity-based protein profiling (ABPP) for enzyme and inhibitor discovery

(**a**) Schematic representation of an activity-based probe. (**b**) A fluorophosphonate (FP) reactive group can be coupled to a tag (e.g., rhodamine, biotin, or alkyne) to covalently label and then detect, enrich, and identify active serine hydrolases. (**c**) In a typical competitive ABPP experiment, a cell or animal model is treated with an inhibitor (or vehicle control), after which proteomes are prepared and incubated with an activity-based probe. SDS-PAGE separation for fluorophore-tagged probes or mass spectrometry analysis of affinity-enriched biotin-tagged probes enables detection and identification, respectively, of the active enzymes in a biological sample. Note that an enzyme that is the target of an inhibitor will show reduced signals in the inhibitor-treated samples relative to vehicle controls ("<").

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Table 1

Human serine hydrolase inhibitors approved for clinical use.

Target	Compound	Structure	Company	Ref(s)	Indication
ACHE	Rivastigmine (Exelon)		Novartis	29	Alzheimer's - associated dementia
	Donepezil (Aricept)		Eisai	69	
	Galantamine (Razadyne)		Ortho-McNeil Janssen	72	
	Tacrine (Cognex)		Shionogi	66	
DPP4	Sitagliptin (Januvia)		Merck	93	Type II diabetes
	Saxagliptin (Onglyza)		Bristol Myers Squibb	92	

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Target	Compound	Structure	Company	Ref(s)	Indication
	Linagliptin (Tradjenta)		Boehringer Ingelheim	94	
	Vildagliptin * (Zomelis)		Novartis	31	
	Alogliptin * (Nesina)		Takeda	86	
Pancreatic/ gastric lipases	Orlistat (Xenical;Alli)		Roche; GlaxoSmithKline	19, 32	Obesity
Thrombin	Dabigatran etexilate (Pradaxa)		Boehringer Ingelheim	46, 47	Thrombosis
	Argatroban (Novastan)		GlaxoSmithKline Mitsubishi Pharma	42	

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Target	Compound	Structure	Company	Ref(s)	Indication
Factor Xa	Rivaroxaban (Xarelto)		Bayer	54, 59	Thrombosis
Human neutrophil elastase	Sivelestat * (Elaspol)		Ono	33	Respiratory disease

The electrophilic moieties of each compound, if applicable, are colored red. The prodrug portions of dabigatran etexilate are colored blue.

* Not yet approved in the United States.

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Table 2

Examples of serine hydrolase targets and lead inhibitors with potential therapeutic value.

Target	Compound (Company, if applicable)	Structure	Ref(s)	Potential Indication	Developmen t Stage
FAAH	OL-135		184	inflammatory pain; nervous system disorders	preclinical
	URB597 (Kadmus Pharmaceuticals)		77		preclinical
	PF-04457845 (Pfizer)		13		Phase II
FAP/ dipeptidyl peptidases	PT-100 (Point Therapeutics)		143, 144	cancer	Phase III (on hold)
LIPG	‘Sulfonylfuran urea 1’ (GlaxoSmithKline)		121	cardiovascular disease	preclinical
PLA2G7	Darapladib (GlaxoSmithKline)		104	atherosclerosis	Phase III
PRCP	‘Compound 8o’ (Merck)		109	obesity	preclinical

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Target	Compound (Company, if applicable)	Structure	Ref(s)	Potential Indication	Developmen t Stage
PREP	S 17092		127	cognitive deficits	preclinical
	JTP-4819 (Japan Tobacco)		126		preclinical
TGH	GR148672X (GlaxoSmithKline)		115	hypertriglycerid- emia	preclinical

The electrophilic moieties of each compound, if applicable, are colored red.

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Table 3

Selective inhibitors recently discovered by competitive ABPP

Target	Compound	Structure	Ref.	Potential Indication(s)
AADACL1	AS115		173	cancer
	JW480		174	
ABHD6	WWL123		11	unknown
ABHD11	AA44-2		14	unknown
	WWL222		11	
APEH	AA74-1		14	unknown
MGLL	JZL184		78	cancer, pain
PAFAH2	AA39-2		14	unknown
PME-1	ABL127		189	cancer, Alzheimer’s disease

The electrophilic moieties of each compound are colored red.